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FoxA3 and goosecoid promote anterior neural fate through inhibition of Wnt8a activity before the onset of gastrulation

Iban Seiliez, Bernard Thisse, Christine Thisse *

Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS/INSERM/ULP, 1, rue Laurent Fries, BP10142, 67404 Illkirch Cedex, France

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Abstract

Formation of the nervous system initially requires the acquisition of neural identity, which is achieved through the inhibition of epidermalizing factors. A regional patterning then takes place within the neural plate through the activity of caudalizing factors. These two processes are tightly regulated early in development by the dorsal organizer. Here, we show that, in zebrafish embryos, two transcription factors, FoxA3 and Goosecoid, coexpressed at the dorsal blastula margin, are required for the definition of anterior neural fate. Their inactivation results in deletions of anterior head structures associated with an increase of Wnt8 activity at the dorsal blastula margin. These phenotypes can be fully rescued by overexpression of Wnt inhibitors or by inactivation of *wnt8a*. Altogether, *foxA3* and *goosecoid* cooperate to promote formation of anterior neural tissue by protecting, as early as blastula stage, presumptive anterior neural cells from an irreversible caudalization by the posteriorizing factor Wnt8a.

Keywords: Goosecoid; *foxA3*; *foxA2*; *dkk1*; *frzb*; *Wnt8a*; Nervous system patterning; Posteriorization; Antero-posterior axis; Zebrafish; Embryo; Blastula

Introduction

The neuroectoderm of the vertebrate gastrula was proposed by Nieuwkoop to be regionalized into forebrain, midbrain, hindbrain and spinal cord by a two-step process. In the activation step, the Spemann gastrula organizer induces neuroectoderm with anterior character, followed with posteriorization by a transforming signal, thereby generating a complete anteroposterior (AP) succession of neural fates (Nieuwkoop et al., 1952). More recently, simultaneous inhibition of BMP and Wnt signaling was shown to induce head formation in frog embryos (Glinka et al., 1997, 1998; Piccolo et al., 1999). Consistent with this, multiple Wnt, Nodal and BMP inhibitors, including Noggin, Chordin, Follistatin, Frzb, Cerberus, Antivin and Dickkopf are expressed in discrete, overlapping domains of the organizer during gastrulation (De Robertis et al., 2000). However, although most of these secreted factors bind to and antagonize the activity of Wnt and/or BMP proteins produced by non-organizer cells, these factors do not repress *wnt* or *bmp* transcription, and are therefore not sufficient to exclude *wnt* and *bmp* expressions from the organizer. As ectopic activation of the zygotic Wnt or BMP pathways inhibits organizer function, a second class of inhibitors may exist which represses the transcription of *wnt* and *bmp* genes in the Spemann organizer. One candidate for such an organizer-specific transcriptional repressor is the homeobox gene *goosecoid* (*gsc*).

Gsc is found across animal phyla, from hydra to human (Blum et al., 1994; Broun et al., 1999; De Robertis et al., 1994; Lemaire and Kodjabachian, 1996). During gastrulation of the vertebrate embryo, *gsc* is expressed in organizer cells—the Spemann organizer in *Xenopus*, the embryonic shield in zebrafish, the node in mouse and chick—and the conservation of Gsc structure and expression suggests an important function in early development (Blum et al., 1992; Blumberg et al., 1991; Izpisua-Belmonte et al., 1993; Schulte-Merker et al., 1994). In *Xenopus*, *gsc* expression peaks at the early gastrula stage in the dorsal mesendoderm that constitutes the Spemann organizer, and injection of ventral blastomeres with *gsc* mRNA induces the formation of a secondary body axis (Cho et al., 1991; Steinbeisser et al., 1993), suggesting that it may be an essential component of the gastrula organizer. Consistent with this idea, overexpression of *gsc* inhibits the expression of *Xwnt8* and

* Corresponding author.
E-mail address: thisse@igbmc.u-strasbg.fr (C. Thisse).

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bmp4 (Christian and Moon, 1993; Fainsod et al., 1994; Steinbeisser et al., 1995), which are expressed in non-dorsal mesoderm, and antagonize organizer function and axis formation (Christian and Moon, 1993; Dale et al., 1992; Fredieu et al., 1997; Hemmati-Brivanlou and Thomsen, 1995; Hoppler and Moon, 1998; Hoppler et al., 1996; Jones et al., 1992, 1996; Tian et al., 1999).

However, additional functional studies in the mouse reveal that knock-out of *gsc* leads to normal embryos and the *gsc*-null mice are born alive. They die soon after birth, however, with craniofacial defects, suggesting that *gsc* is not essential for organizer activity but that it is required later during embryogenesis for craniofacial and rib development (Belo et al., 1998; Rivera-Pérez et al., 1995; Yamada et al., 1995, 1997; Zhu et al., 1998). Given that organizer expression of *gsc* is absolutely conserved in vertebrates, the lack of an early phenotype suggests that functionally redundant genes are expressed in the gastrula embryo which compensate for the loss of *gsc* function.

To investigate this hypothesis, we searched for potential candidates by the use of a large-scale in situ screen we are currently performing in the aim of identifying the expression pattern of the whole zebrafish genome. Among our collection of 14,000 different genes analyzed, only two of them, *gsc* and *foxA3*, shared the same expression pattern at early developmental stages, in both the dorsal marginal blastoderm and in the dorsal marginal part of the yolk syncitial layer. FoxA3 belongs to the Hepatocyte Nuclear Factor 3 (HNF3 or FoxA) family, known to play a crucial role in the regulation of metabolism and in the differentiation of tissues such as pancreas and liver (Kaestner, 2000). Nevertheless, no information was available about its possible function during early developmental stages except a partial description of its expression pattern at early stages (Odenthal and Nüsslein-Volhard, 1998). We therefore investigated its potential role during early development by inhibiting its activity using the morpholino knock-down technology. We also reexamined in zebrafish the function of *gsc* using the same strategy and tested by double knock-down the potential redundant function of these genes during early developmental stages. In this report, we show that *foxA3* and *gsc* cooperate to promote formation of anterior neural tissue by protecting presumptive anterior neural cells from the caudalizing activity of Wnt8a. We further demonstrate that rostral presumptive neural tissues need to be protected from Wnt8a stimulation very early in development, as early as blastula stage, to prevent their irreversible caudalization by this posteriorizing factor.

Materials and methods

Whole-mount in situ hybridization

All whole-mount in situ hybridizations were performed as described previously (Thisse et al., 2004a). *foxA3*, *frzb*, *dkk1*, *foxi1*, *pax2.1*, *hoxa1*, *otx5* and *pax6* were isolated in the course of a large-scale in situ hybridization screen (http://zfin.org) and antisense RNA made through *Not*I linearization and T7 transcription. The other clones used in this study have been previously described: *gsc* (Thisse et al., 1994), *emx1* (Morita et al., 1995), *eng2* (Ekker et al., 1992) and *wnt8a* (Kelly et al., 1995).

DNA, mRNA and morpholino injections

The *wnt8a* (a gift of R. Moon) and *dkk1* ORFs were subcloned into *Bam*HI/*Xho*I sites of pCS2+. The *gsc* and *frzb* ORFs were subcloned into the *Eco*RI/*Xho*I sites of pCS2+. Plasmids were then linearized with *Not*I and sense RNA transcribed with SP6 RNA polymerase using the mMessage mMachine Kit (Ambion).

Morpholinos (Gene Tools) were resuspended in Danieau 1×, stored at −20°C as a 4 mM stock solution and diluted before use to the appropriate concentration. The sequences of the morpholinos used are:

Mo-gsc: CAAGCGAAAAGATGTGTGAGATTTG
Mo-gsc(D): AGTAAAAAAATACCTGTAGGAATAC
Mo-gsc(A): GCGCTGTCATAACCTGAAAATAAGA
Mo-foxA3: CTCGTAAGAAACGGGATAGTGACTG
Mo-dkk1: GAGAGCATGGCGATGTGCATCATGT
Mo-wnt8a: ACGCAAAAATCTGGCAAGGGTTCAT
Mo-foxA2: CCTCCATTTTGACAGCACCGAGCAT

For mRNA and morpholino injections, embryos were dechorionated using Pronase E and injected with either RNA or morpholinos diluted in 0.2% Phenol Red and 0.1 M KCl, using an Eppendorf 5426 microinjector.

Cells transplantation

Transplantation experiments were performed by suction as described (Saùde et al., 2000). Donor embryos were injected at the 1–2 cell stage with dkk1 plus GFP RNA (100 and 50 pg, respectively) or GFP RNA alone (50 pg) as a control. Host embryos were injected at the 1–2 cell

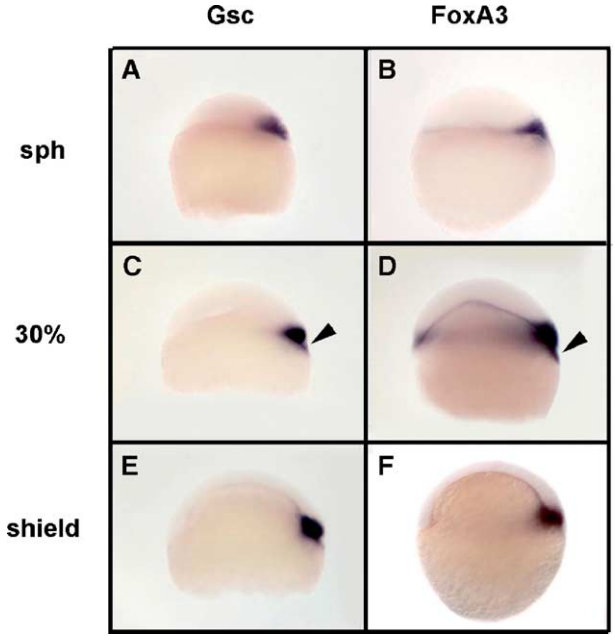
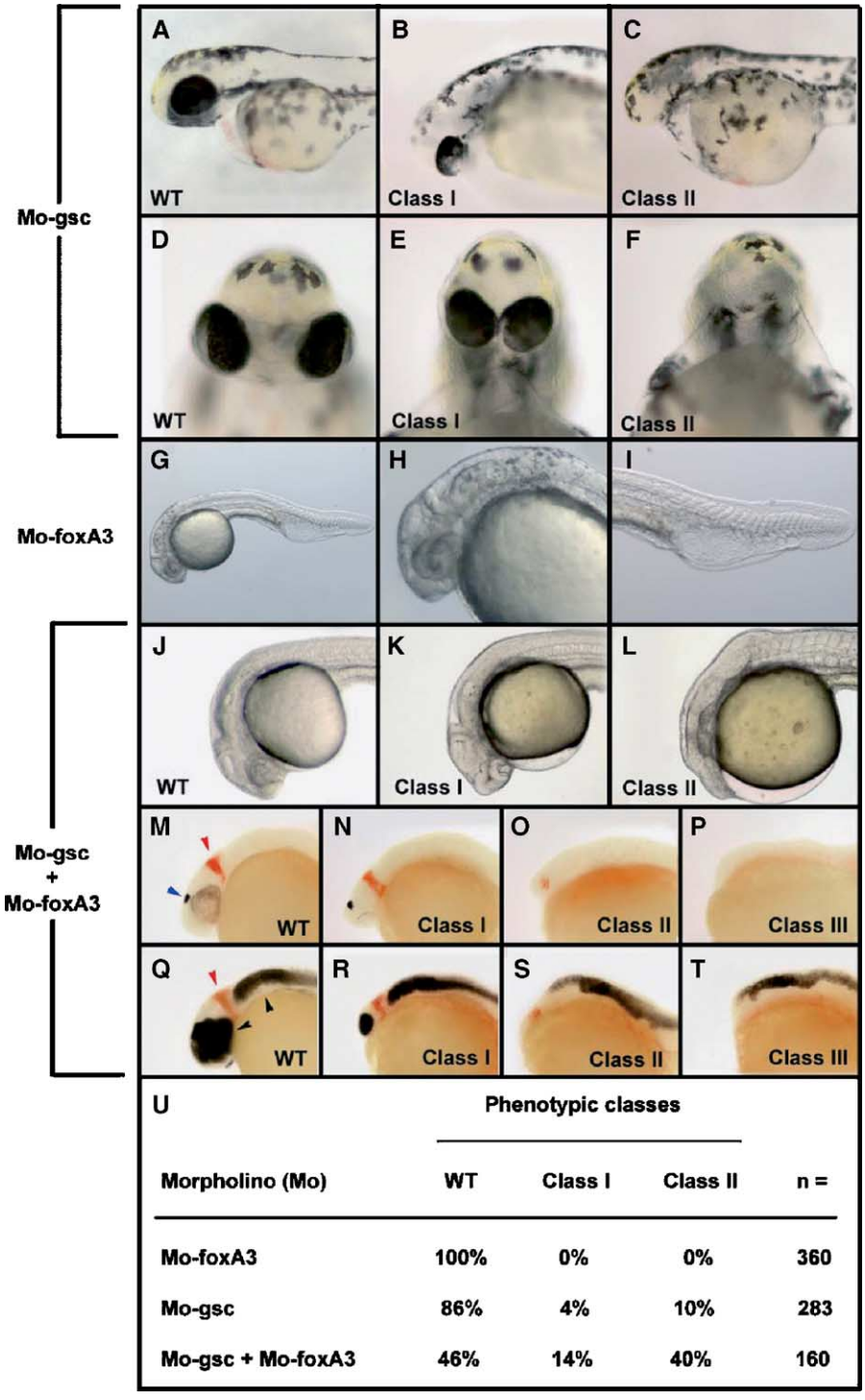


Fig. 1. Expression of gsc and foxA3 during early developmental stages. (A, B) At sphere stage, *gsc* and *foxA3* are expressed at the dorsal margin. (C, D) At blastula stage (30% epiboly), *gsc* and *foxA3* transcripts colocalize at the dorsal margin with strong expression in blastodermal cells and YSL (arrowhead). (E, F) At the onset of gastrulation, *gsc* and *foxA3* are expressed in the central part of the embryonic shield. Transcripts of both genes are no longer observed in the YSL. Embryos are in lateral view, dorsal towards the right.

stage with gsc and foxA3 morpholinos (20 and 10 pg, respectively). Cells were transplanted from the animal pole of a donor to the animal pole of a host embryo in 1× Danieau medium supplemented with 2% penicillin/streptomycin. Transplantation needles (pulled from borosilicate glass capillaries, Harvard Apparatus GC120F-15) were cut to a diameter of 80 μm. The number of grafted cells was in between 200 and 300. Embryos were cultured in small Petri dishes in Danieau 0.3× medium supplemented with 2% penicillin/streptomycin at 28.5°C.



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Morpholino (Mo)	Phenotypic classes: WT	Class I	Class II	n =
Mo-foxA3	100%	0%	0%	360
Mo-gsc	86%	4%	10%	283
Mo-gsc + Mo-foxA3	46%	14%	40%	160

Fig. 2. *foxA3* inactivation enhances *gsc* loss-of-function phenotype. (A–F) Inactivation of *gsc* leads to head structure alterations ranging from cyclopia (class I, B and E) to a complete deletion of the anterior head (class II, C and F). (G–I) Morpholino knock-down of *foxA3* results in an expansion of ventro-caudal mesenchyme (G and I) whereas the head is unaffected (H). (J–T) Double inactivation of *gsc* and *foxA3* results in head truncation visible morphologically as a cyclopia (class I, K) or a complete deletion of anterior head (class II, L). Analysis after in situ hybridization with *otx5* (epiphysis, blue, M–P) and *eng2* (MHB, red, M–P) or (Q–T) with *pax6* (eye, forebrain, blue) and *eng2* (red) reveals much stronger phenotypes including complete loss of anterior structures from telencephalon up to MHB (class III). (U) The penetrance of class I and class II phenotypes analyzed at 24 hpf in Mo-gsc plus Mo-foxA3 injected embryos (20 pg each Mo) was also strongly enhanced compared to the Mo-foxA3 (20 pg) or Mo-gsc (20 pg) injected groups. (A–C; G–T) Lateral view, (D–F) front view. (A–F) Embryos at 36 hpf; (G–T) embryos at 24 hpf.

Results

Correlation of foxA3 and gsc expression patterns during early developmental stages

We identified the helix–wing–helix transcription factor FoxA3 during the course of a large-scale in situ hybridization screen designed to characterize the spatial and temporal expression pattern of the whole zebrafish genome during embryonic development (Thisse et al., 2004a). Its expression at blastula and gastrula stages strikingly correlated with the expression pattern of *gsc* at these early developmental stages (Schulte-Merker et al., 1994; Stachel et al., 1993; Thisse et al., 1994). Transcripts of both *foxA3* and *gsc* are first detectable by in situ hybridization at sphere stage, at the dorsal margin of the blastoderm (Figs. 1A and B and Odenthal and Nüsslein-Volhard, 1998). During late blastula stage, *foxA3* and *gsc* transcripts colocalize at the dorsal margin in both blastodermal cells and yolk syncytial layer (YSL, Figs. 1C and D). As gastrulation begins, all *foxA3* and *gsc*-expressing cells invaginate and form the central part of the embryonic shield (Figs. 1E and F) that will become the leading edge of the mesendoderm, which extends anteriorly under the ectoderm during the course of gastrulation.

Among the 14,000 different genes screened so far, only *foxA3* and *gsc* displayed such an expression pattern at early developmental stages suggesting potential interactions between the two corresponding proteins.

foxA3 inactivation enhances gsc loss-of-function phenotype

We probed *foxA3* and *gsc* activity and tested their possible relationships by loss-of-function experiments using morpholino (Mo) knock-down technology. Injection of a morpholino directed against the translation initiation site of *gsc* (see Materials and methods) had only a weak effect on zebrafish development. Injection of high amount of Mo-gsc (up to 20 pg) led to 86% of embryos indistinguishable from wild-type (Figs. 2A and D). However, the remaining 14% of embryos displayed head structure alterations ranging from cyclopia (4%—class I, Figs. 2B and E) to a complete deletion of the rostral part of the head (10%—class II, Figs. 2C and F). In contrast, *foxA3* knock-down embryos (injection up to 20 pg Mo) displayed a weak expansion of the ventro-caudal mesenchyme at 24 hpf (Fig. 2I) but showed no head abnormalities (Figs. 2G and H). Therefore, conversely to *gsc*, *foxA3* appears not to be involved in head formation. Nevertheless, this does not exclude a possible functional redundancy between these two genes during early developmental stages.

To investigate their possible cooperation, we performed morpholino double inactivation. As shown in Figs. 2J–L, inactivation of *gsc* plus *foxA3* (20 pg Mo, respectively) results

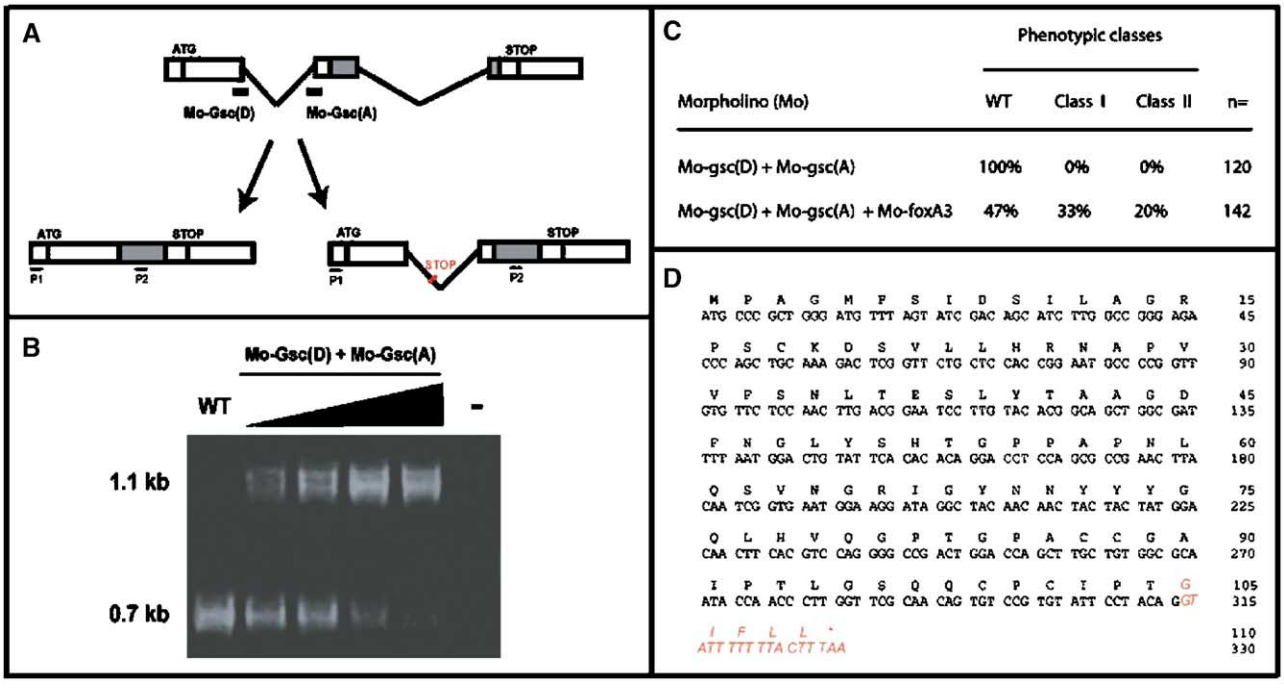


Fig. 3. Maternal and zygotic contribution of *gsc* in the headless phenotype. (A) Correct and aberrant splicing of zygotic *gsc* pre-mRNA in the presence of morpholinos directed against the donor, Mo-gsc (D), and the acceptor, Mo-gsc (A), of splice of the first intron of the gsc gene. Exons are boxed, homeobox is in grey. P1 and P2, forward and reverse primers used for RT-PCR analysis of injected embryos. (B) RT-PCR analysis of progressive inhibition of the splicing of gsc first intron in embryos injected with a range of Mo-gsc (D) and Mo-gsc (A). The numbers on the left indicate the size of the RT-PCR products representing the aberrantly (1.1 kb) and correctly (0.7 kb) spliced RNAs. Lane 1, non-injected wild-type embryos; lanes 2–5, embryos injected with 0.6 pg, 1.2 pg, 2.5 pg and 5 pg of each morpholino, respectively; lane 6, reverse transcriptase free negative control. (C) Frequency of class I and class II phenotypes analyzed at 24 hpf in Mo-gsc (D) plus Mo-gsc (A) injected embryos (5 pg each Mo) and in embryos with an additional injection of Mo-foxA3 (10 pg). (D) Nucleic and amino acid sequence of gsc fragment generated from the aberrant splicing. Intronic region is shown in red and italic. Star indicates the stop codon.

in anterior head deletion similar to single inactivation of *gsc*. However, the penetrance of phenotypes was strongly enhanced with 14% of cyclopic embryos (class I) and 40% of embryos displaying anterior head deletion (class II, Fig. 2U). Further characterization of *gsc/foxA3* knock-down embryos by in situ hybridization using specific probes of anterior head (*otx5* and *eng2*, Figs. 2M–P, or *pax6* and *eng2*, Figs. 2Q–T) revealed that head alterations included 25% (7/29) of cyclopic embryos (class I), 48% (14/29) individuals with complete lack of forebrain and midbrain (class II) and 27% (8/29) of the embryos (class III) with additional loss of midbrain–hindbrain boundary (MHB), a phenotype which was never observed for the single inactivation of *gsc*.

Altogether, these results show that *foxA3* inactivation strongly enhances gsc loss-of-function phenotype and reveals a functional redundancy of these two genes for head development.

Maternal and zygotic contributions of gsc for anterior head formation

In previous studies, Gsc has been shown to be both maternally and zygotically expressed (Schulte-Merker et al., 1994; Stachel et al., 1993; Thisse et al., 1994). Morpholino directed against the initiation of translation site prevents translation from both maternal and zygotic mRNA. In order to distinguish between maternal and zygotic functions of *gsc*, we designed morpholinos directed against donor and acceptor of splice of the first intron of the *gsc* gene (Fig. 3A). Because these morpholinos prevent splicing, they will only affect zygotic transcript while the translation of maternal mRNA will be unaffected.

Injection of increasing doses of morpholinos directed against the acceptor and donor of splice results in a progressive inhibition of the splicing for the first intron. Analysis by RT-PCR revealed that injection of 5 pg of each Mo resulted in a nearly complete inhibition of zygotic *gsc* expression (Fig. 3B). Nevertheless, all injected embryos displayed wild-type head (Fig. 3C). This result suggests that the zygotic *gsc* function is not required for head formation. In such conditions, we expected that additional inactivation of *foxA3* should not perturb head development. To our surprise, injection of 10 pg Mo-foxA3 together with Mo-gsc(D) and Mo-gsc(A) resulted in head truncation phenotypes comparable to inactivation of *foxA3* and maternal-zygotic *gsc* functions (Fig. 3C). This indicates that induction of the head depends on the interaction between *foxA3* and zygotic *gsc*.

However, sequence analysis showed that the peptide translated from *gsc* transcripts containing the first intron sequence results in a truncated protein containing the first 104 amino acids of the protein followed by 5 amino acids translated from the intron sequence but lacking the carboxy-terminal half of Gsc including its homeodomain (Fig. 3D). To demonstrate that the headless phenotype observed is not due to a dominant negative activity carried by this truncated form of Gsc, we injected a synthetic mRNA coding for this peptide into one cell stage embryos. Injection of up to 1 ng of sense RNA does not affect embryonic development demonstrating that this peptide has no biological activity (data not shown).

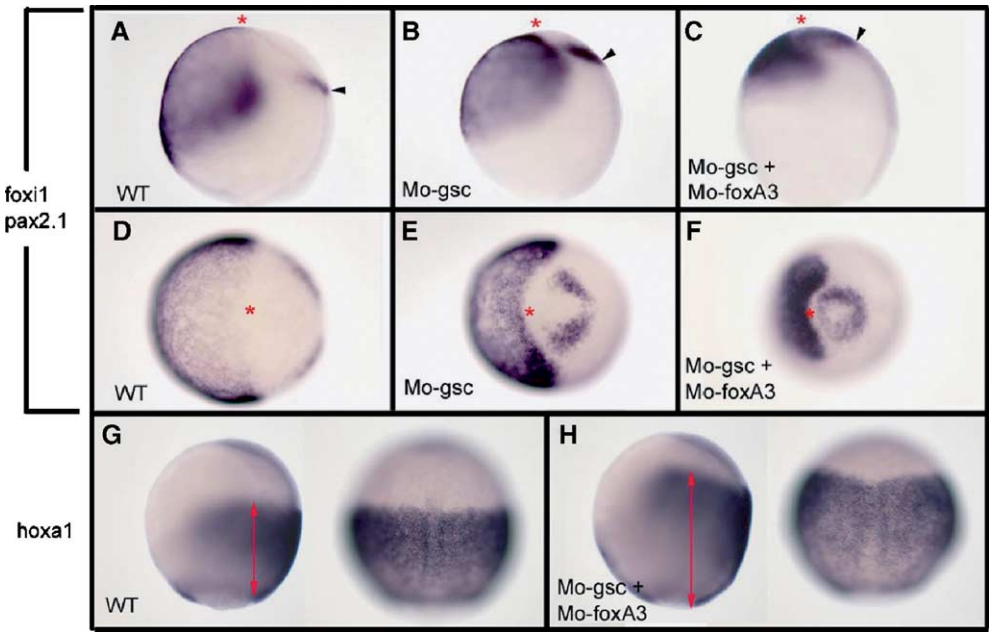


Fig. 4. Inhibition of *gsc* plus *foxA3* posteriorizes the ectoderm. (A–F) Expression of the presumptive epidermal marker *foxi1* and the presumptive MHB marker *pax2.1* in WT (A, D), *gsc* (B, E) and *gsc/foxA3* (C, F) knock-down embryos. The region between the animal pole (red star) and the presumptive MHB (arrowhead) corresponding to the presumptive forebrain and midbrain is reduced in *gsc* and almost absent in *gsc/foxA3* knock-down embryos. (G, H) Expression of *hoxa1* in WT (G) is expanded anteriorly (red arrow) in *gsc/foxA3* knock-down embryos (H). The embryos are at 80% epiboly. (A–C, G and H left) Lateral views and (D–F) animal pole view, dorsal to the right. (G and H right) Dorsal views anterior to the top.

Overall, these results demonstrate that *foxA3* acts synergically with the zygotically expressed *gsc* for the formation of anterior head structures.

Inhibition of gsc plus foxA3 leads to reduction of anterior neural plate at gastrula stage

We showed that *foxA3* inhibition enhances gsc phenotype at 24 hpf (Fig. 2). However, *foxA3* and *gsc* are coexpressed at blastula stage suggesting that their synergistic interactions occur early in development. To investigate this, we compared *gsc* and *gsc/foxA3* knock-down embryos at gastrula stage for their expression of *foxi1* and *pax2.1*, specific of presumptive epidermis and presumptive MHB, respectively (Figs. 4A–F). Presumptive forebrain and midbrain, delimited by epidermis and MHB, appear reduced in *gsc* knock-down embryos and this phenotype is strongly enhanced for double *gsc/foxA3* inactivation (Figs. 4A–F). Presumptive epidermal territory appears also reduced, expression remaining only detectable at the animal pole for *gsc/foxA3* double inactivation. Accordingly, the posterior neural plate expands anteriorly as shown by the extension of *hoxa1* expression along the animal–vegetal axis (Figs. 4G and H). Therefore, these results reveal that loss of *gsc* plus *foxA3* functions results in a posteriorization of the ectodermal territory as early as gastrulation stage.

Inhibition of gsc and foxA3 results in an upregulation of Wnt8 signaling

Previous studies have shown that upregulation of Wnt8 signaling results in a loss of anterior head development (Kelly et al., 1995). As exemplified in Fig. 5B, injection of a DNA construct containing *wnt8a* ORF under the control of a constitutive promoter (100 ng/µl) results into anterior head truncation. Furthermore, an upregulation of Wnt8 signaling pathway through inactivation of the Wnt antagonist *dkk1* (injection of 20 pg Mo-dkk1) causes anterior head truncation (Fig. 5C). This strongly suggests a relationship between *foxA3*, *gsc* and the Wnt8 signaling pathway for the formation of anterior head. To probe this hypothesis, we examined the expression of *wnt8a* as well as *frzb* and *dkk1*, two Wnt antagonists, in *gsc* and in *gsc/foxA3* knock-down embryos. Inactivation of *gsc* resulted in the dorsal expansion of *wnt8a* expression at the blastula margin (Fig. 5E compared to D) that

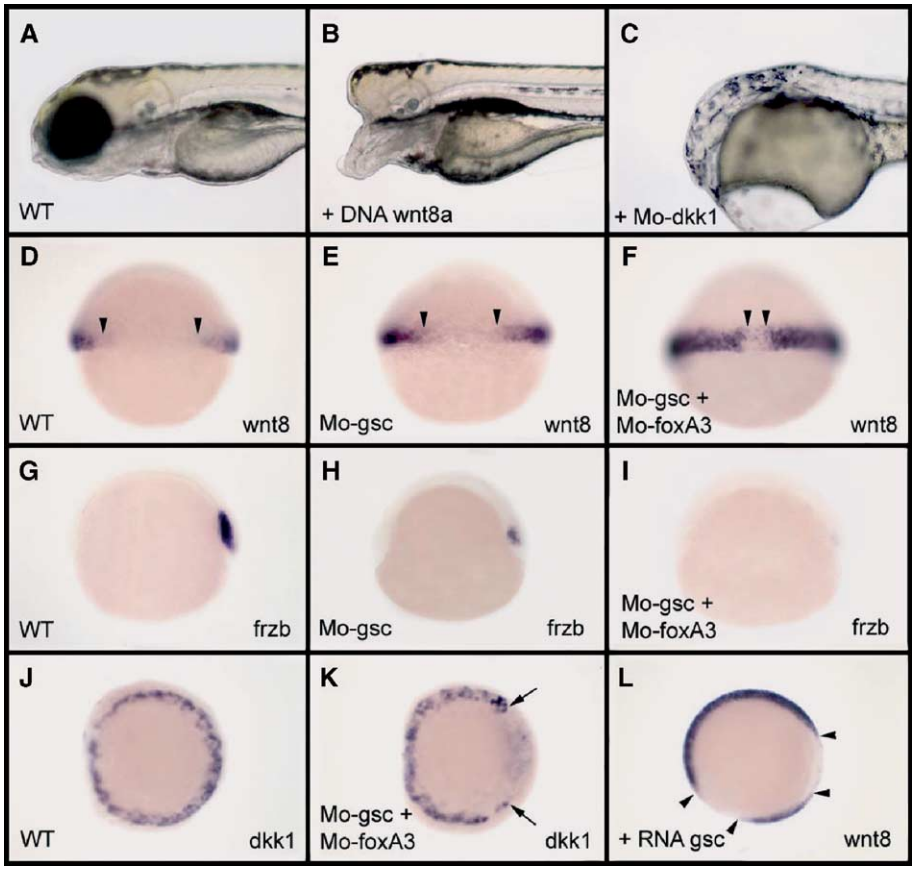


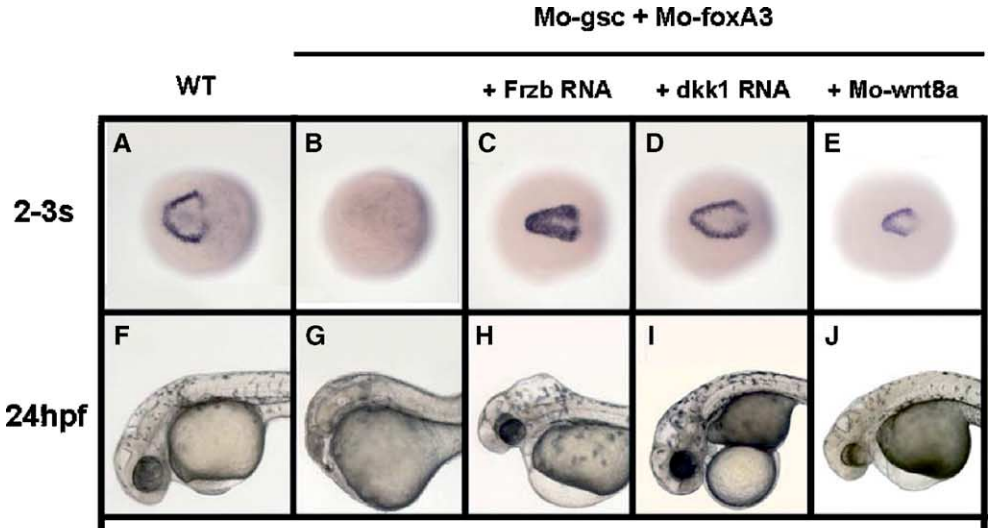
Fig. 5. Inhibition of *gsc* and *foxA3* results in an upregulation of Wnt signaling at the dorsal margin. Compared to wild-type (A), embryos injected with *wnt8a* DNA (B) or inactivation of *dkk1* by morpholino knock-down (C) results in deletion of anterior head. (D–F) Expression of *wnt8a* at shield stage in WT (D), *gsc* (E) and *gsc*/*foxa3* (F) knock-down embryos. Arrowheads indicate the dorsal borders of *wnt8a* expression territory. (G–I) Expression of *frzb* at shield stage in WT (G), *gsc* (H) and *gsc*/*foxa3* (I) knock-down embryos. (J, K) Expression of *dkk1* at shield stage in WT (J) and *gsc*/*foxa3* knock-down (K) embryos. Arrows indicate the dorsal most extent domain of *dkk1* expression at the margin. (L) Expression of *wnt8a* at shield stage in an embryo injected with *gsc* RNA at the 32 cell stage in one ventro-lateral blastomere. Arrowheads indicate the limit of wnt8a expression at the gastrula margin. (A, B) 96 hpf, (C) 48 hpf, (D–L) shield stage. (A–C, G–I) Lateral view, (D–F) dorsal view, (J–L) animal pole view dorsal to the right.

was even more pronounced for double inactivation of *gsc* and *foxA3* (Fig. 5F). Conversely, expression of *frzb* was reduced in *gsc* and completely lost in *gsc/foxA3* knock-down embryos (Figs. 5G–I). Similarly, expression of *dkk1* disappeared from the dorsal margin of *gsc/foxA3* knock-down embryos (Figs. 5J and K). This demonstrates that loss of *gsc* and *foxA3* function results in an upregulation of *wnt8* activity at the dorsal margin due to the dorsal expansion of *wnt8a* expression domain and the concomitant loss of dorsal Wnt inhibitors. Involvement of *gsc* in this process is further supported by the inhibition of *wnt8a* expression at the ventral margin following a local misexpression of *gsc* (Fig. 5L).

Altogether, these observations strongly suggest that the loss of head structures in *gsc* or *gsc/foxA3* knock-down embryos is due to an increase of *wnt8* activity at the dorsal gastrula margin.

Rescue of head formation through inhibition of wnt8 activity

We hypothesized that head alterations in *gsc/foxA3* knock-down embryos resulted from an increase of Wnt8 signal at the



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at 2-3 somite stage:	Phenotypic classes (emx1 expression)			
Injections	WT	reduced expression	no expression	n =
Mo-gsc + Mo-foxA3	34%	36%	20%	59
Mo-gsc +Mo-foxA3 + frzb RNA	89%	11%	0%	49
Mo-gsc + Mo-foxA3 + dkk1 RNA	100%	0%	0%	37
Mo-gsc + Mo-foxA3 + Mo-wnt8a	75%	18%	7%	60

at 24 hpf:	Phenotypic classes			
Injections	WT	cyclopia	anterior head truncation	n =
Mo-gsc + Mo-foxA3	13%	16%	71%	31
Mo-gsc +Mo-foxA3 + frzb RNA	42%	27%	31%	204
Mo-gsc + Mo-foxA3 + dkk1 RNA	86%	10%	3%	86
Mo-gsc + Mo-foxA3 + Mo-wnt8a	58%	26%	16%	50

Fig. 6. Inhibition of Wnt8a signaling rescues the headless phenotype of *gsc/foxA3* knock-down embryos. Expression of the telencephalic marker *emx1* in wild-type embryo (A), in double *gsc/foxa3* knock-down embryo (B), in *gsc/foxa3* knock-down embryo coinjected with either frzb RNA (C), dkk1 RNA (D) or wnt8a Mo (E). (F–J) Morphological analysis of embryos treated with the same conditions of injections than in panels A–E showing rescue of the head at 32 hpf when the Wnt signaling pathway is inhibited. (K) Frequency of the phenotypic classes observed for the abovementioned injections. (A–E) 2–3 somite stage, dorsal views; (F–J) lateral views at 32 hpf.

dorsal margin. If this is true, then the blockage of Wnt signaling pathway should rescue the headless phenotype. In order to assess this, frzb or dkk1 RNA (1 ng and 100 pg, respectively) was coinjected with gsc/foxA3 morpholinos into two-cell stage embryos. Half of the injected embryos were grown until the 2–3 somite stage, fixed and analyzed by in situ hybridization for the expression of the telencephalon marker *emx1* (Figs. 6A–D). The rest of the embryo population was grown up to 32 hpf for morphological analyses (Figs. 6F–I).

As described in Fig. 2, injection of gsc/foxA3 Mo (20 and 10 pg, respectively) results in severe head truncation at 32 hpf (Figs. 6G and K). At the 2–3 somite stage, half of the injected embryos (56%, Fig. 6K) show strong reduction or absence of *emx1* expression (Fig. 6B compared to A). In contrast, injection of frzb RNA (1 ng) together with gsc/foxA3 morpholinos (20 and 10 pg, respectively) leads to rescue the headless phenotype (Figs. 6H and K) with 89% of embryos showing a strong rescue of *emx1* expression (Figs. 6C and K). Similar results were obtained after coinjection of dkk1 RNA (100 pg). In this case, 100% of injected embryos show a restored *emx1* expression at the 2–3 somite stage (Figs. 6D and K) and display a well developed head at 32 hpf (Figs. 6I and K). These results clearly demonstrate that the loss of anterior head structures in *gsc/foxA3* knock-down embryos is due to an increase of Wnt signaling.

Because gsc/*foxA3* knock-down results in a dorsal expansion of *wnt8a* expression, we hypothesized that *gsc* and *foxA3* promote anterior head formation by preventing expression of *wnt8a* at the dorsal margin. We then performed additional inactivation of *wnt8a* (injection of 15 pg Mo-wnt8a) in double *gsc/foxA3* knock-down embryos. As for the coinjection of Wnt inhibitors, additional inhibition of *wnt8a* rescues the formation of anterior neural tissue in *gsc/fox3* morphants resulting in restoration of *emx1* expression at the 2–3 somite stage and to a well developed head at 32 hpf (Figs. 6E, J and K).

Altogether, these results demonstrate that formation of anterior head depends on an inhibition of Wnt activity at the dorsal margin and that *gsc* and *foxA3* cooperate to repress *wnt8a* expression at early developmental stages.

gsc and foxA3 promote anterior neural development by limiting wnt8a activity as early as blastula stage

Our results show that *gsc* and *foxA3* promote rostral head structure development by inhibiting *wnt8a* activity at the dorsal margin (Fig. 6). In this regard, their expression in the embryonic shield correlates well with the known axis inducing the property of the zebrafish organizer which is characterized by anti-wnt and anti-bmp activities (De Robertis et al., 2000). However, *foxA3* and *gsc* are already coexpressed at blastula stage (Fig. 1) (Schulte-Merker et al., 1994; Stachel et al., 1993; Thisse et al., 1994). This suggests that they may fulfil their Wnt signaling inhibitory activity at the dorsal margin well before the onset of gastrulation. We probed this hypothesis by examining the timing of *wnt8a* activity through the analysis of stage-specific rescue experiments. We transplanted a group of cells secreting the strong Wnt inhibitor Dkk1 at the animal pole of *gsc/foxA3* double knock-down embryos (Fig. 7A, see also Materials and methods) at two different stages, either at sphere (early blastula stage) or shield stage (onset of gastrulation). The host embryos were then grown until the 2–3 somite stage and probed for the expression of *emx1* or were grown up to 24 hpf for a morphological analysis (Figs. 7B–I). Whereas grafts of animal pole cells from control donor embryos had no effect on the *gsc/foxA3* knock-down phenotype (Figs. 7C, G and L), grafts of Dkk1 expressing cells at the sphere stage strongly rescue the headless phenotype. 63% of grafted embryos displayed wild-type like *emx1* expression at 2–3 somite stage and 68% of the embryos had a well shaped head at 24 hpf (Figs. 7B, F and L). In contrast, when grafts of Dkk1 expressing cells were performed at the shield stage, the results were comparable to control experiments and we failed to observe any rescue of anterior neural tissue (Figs. 7D, H and L). Therefore, while grafts of cells expressing a Wnt inhibitor at blastula stage are able to strongly rescue *gsc/foxa3* phenotype, grafts at the shield stage are unable to rescue the headless phenotype resulting from *gsc/foxa3* inactivation. Notably, embryos grafted at the shield stage lack ventral mesoderm (Fig. 7J). This demonstrates that Dkk1 secreted by the grafted cells is able to efficiently inhibit *wnt8a* activity at long distance (from the animal pole to the ventral margin). This effect on the ventral margin territory can be used as an internal control of Dkk1 activity and further proves that inhibition of Wnt activity at the onset of gastrulation cannot rescue head induction anymore.

In consequence, these results clearly demonstrate that, at the onset of gastrulation, cells already stimulated at blastula stage by Wnt8a are irreversibly posteriorized. This supports the idea that *gsc* and *foxA3* function at blastula stage, before the onset of gastrulation, to prevent stimulation of presumptive anterior neural tissues by the caudalizing factor Wnt8a.

Discussion

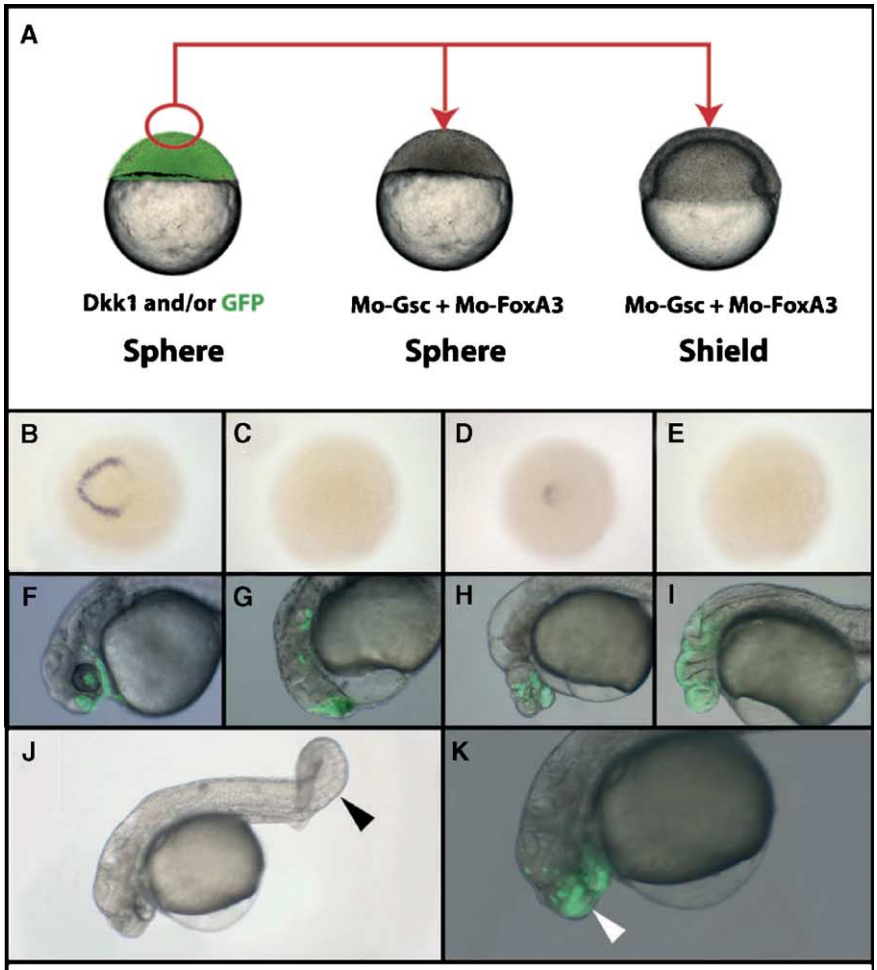
Synergistic interaction of gsc and foxA3 for the anterior neural plate formation

The most striking feature of *foxA3* expression is that it closely matches with that of *gsc* as soon as the blastula stage at the dorsal margin in both blastodermal cells and yolk syncycial layer suggesting potential functional interactions between these two genes at early developmental stages. To probe this hypothesis, we compared phenotypes of double gsc/*foxA3* knock-down to phenotypes resulting from single inactivation of *gsc* or *foxA3*.

We show that single *gsc* loss-of-function results in alteration of head structures ranging from cyclopia to a complete deletion of rostral head (Fig. 2). However, we observed a rather low penetrance of these phenotypes (Fig. 2U). These results are comparable to those obtained in *Xenopus* using antisense RNA injection (Steinbeisser et al., 1995). Similarly, injection of antimorphic Gsc results in embryos showing anterior head defects. However, at the contrary of morpholino knock-down phenotypes, these embryos displayed additional axial and gastrulation abnormalities (Ferreiro et al., 1998; Latinkic and Smith, 1999;

Yao and Kessler, 2001). This may result from differences between the mechanisms of action of morpholinos and antimorphic proteins. Morpholinos lead to a strong, specific decrease in gsc activity. Because Gsc acts as a transcriptional repressor, this results in a stage and tissue-specific derepression of Gsc-target genes. However, overexpression of antimorphic proteins strongly and constitutively activates these genes in all cells of the embryo.

Conversely to single *gsc* inactivation, *foxA3* loss-of-function does not affect head development but it strongly



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at sphere stage :	WT	cyclopia	anterior head truncation	n =
	Phenotypic classes			
Mo-gsc +Mo-foxA3	21%	36%	43%	14
+ dkk1 RNA and GFP RNA	68%	11%	21%	19
+ GFP RNA	0%	31%	69%	13

at shield stage :	WT	cyclopia	anterior head truncation	n =
	Phenotypic classes			
Mo-gsc +Mo-foxA3	7%	51%	42%	181
+ dkk1 RNA and GFP RNA	21%	43%	36%	14
+ GFP RNA	36%	27%	36%	11

enhances the penetrance of *gsc* loss-of-function phenotype. In addition, double inactivation of *gsc/foxA3* results in additional loss of MHB, a phenotype never observed for the single inactivation of *gsc* (Fig. 2). This result clearly establishes that these two genes interact and cooperate to promote formation of anterior neural structures.

A cooperative interaction between *gsc* and another fork-head domain family protein, HNF3β (FoxA2), has been reported in the mouse (Filosa et al., 1997). Embryos heterozygous for *hnf3β* and homozygous mutants for *gsc* showed a dramatic reduction in forebrain size, a phenotype not observed in *hnf3β* and *gsc* single mutants (Ang and Rossant, 1994; Rivera-Pérez et al., 1995; Weinstein et al., 1994; Yamada et al., 1995). This suggested that FoxA2 may also interact with goosecoid in fish. Nevertheless, zebrafish FoxA2 (HNF3β, Axial) is expressed later than FoxA3 and with a different expression pattern (excluded from the prechordal plate but expressed in endoderm) making less likely that it synergizes with gsc. Even so, we functionally probed the possible redundancy of FoxA2 and FoxA3 by performing double morpholino knock-down of these two genes. Using the same morpholino sequences and the same doses as previously described for FoxA2 loss of function (Norton et al., 2004), we generated a phenotype identical to *monorail* (corresponding to the *foxA2* mutation). The double inactivation of FoxA2 and FoxA3 did not result in head defects showing that *foxA2* is not redundant to *foxA3* for the formation of the head. Similarly, double inactivation of *foxA2* and *gsc* did not enhance the frequency of anterior head structure deletions observed in the context of *gsc* single inactivation. This demonstrates that *foxA2* is not redundant to *foxA3* in its interaction with *gsc* and in the control of anterior head formation.

Finally, a third member of the same fork-head domain subgroup, FoxA1 (FoxA1/HNF3α), has been reported in zebrafish, but its expression (Odenthal and Nüsslein-Volhard, 1998; Thisse et al., 2004b, gene expression section of the Zebrafish International Network Database- zfin.org) starts too late (bud stage) and its territories of expression (notochord, hypochord and floor plate) do not overlap with those of *gsc* and *foxA3* making unlikely that *foxA1* plays a redundant function with *foxA3* for an interaction with *gsc* in the head formation.

gsc and foxA3 act through the regulation of wnt8a activity

In the early gastrula, *wnt8a* expression in the marginal zone is complementary to the *gsc* and *foxA3* expression domains (Lekven et al., 2001) (Figs. 1 and 5D) and the upregulation of Wnt8 signaling results in a loss of anterior head development (Kelly et al., 1995) (Figs. 5B and C). These two observations suggested a possible relationship between *foxA3*, *gsc* and the Wnt8a signaling pathway for the formation of anterior head. Accordingly, we showed that *foxA3/gsc* inactivation induces dorsal expansion of *wnt8a* expression and simultaneously reduces the dorsal expression of the Wnt inhibitors *frzb* and *dkk1* (Figs. 5D–K). This upregulation of Wnt activity results in anterior head deletions similar to defects observed following zygotic *wnt8* overexpression (injection of a DNA construct expressed after the midblastula transition). Further proof of the implication of Wnt8 in *gsc/foxA3* morphants phenotype is that the head deletion can be fully rescued by overexpression of *frzb* or *dkk1* as well as by morpholino inactivation of *wnt8a* (Fig. 6).

A *wnt8* repressing function for *gsc* was described in *Xenopus* (Steinbeisser et al., 1995; Yao and Kessler, 2001). This effect is further supported by the identification of multiple Gsc-binding sites in the *Xwnt8* promoter demonstrating its potential for direct repression of *wnt8* transcription (Yao and Kessler, 2001). However, data from *Xenopus* differ significantly from ours in that the injection of antimorphic Gsc does not alter the expression of *frzb* (Yao and Kessler, 2001). This may result from inter-species differences in early developmental processes.

Altogether, we show here that in zebrafish the cooperative function of *gsc* and *foxA3* leads to anterior head development not only by suppressing the transcription of *wnt8a* at the dorsal margin but also by promoting the dorsal expression of *frzb* and *dkk1*. This last effect on these two secreted wnt antagonists could contribute significantly in the well characterized non-cell-autonomous effect of *gsc* axis inducing activity (Cho et al., 1991). Finally, the strong enhancement of *gsc* loss-of-function phenotype by *foxA3* inactivation suggests an involvement of this transcription factor in this process that has never been described before.

gsc and foxA3 function as early as blastula stage to prevent exposure of anterior presumptive neural plate to Wnt8a activity

Explant and transplant studies in amphibian, mouse, chick and fish embryos indicate that neural determination has commenced by midgastrula stages (Ang et al., 1994; Holtfreter and Hamburger, 1955; Jones and Woodland, 1989; Roberts et al., 1991; Sagerström et al., 1996; Sive et al., 1990). Analysis using molecular markers is making it clear that, even before midgastrula, the presumptive neuroectoderm has begun to be

Fig. 7. Rescue of headless phenotype by transplantation of Dkk1 expressing cells. (A) Scheme describing the experimental procedures. A group of cells from sphere stage embryos injected at the one cell stage with either dkk1 plus GFP RNA or GFP RNA alone was grafted at the animal pole of *gsc/foxA3* knock-down embryos either at sphere or at shield stage. Host embryos were grown up to the 2–3 somite stage and were analyzed for the expression of *emx1* or grown up to 24 hpf for morphological analyses. (B–E) *emx1* expression in 2–3 somite stage embryos grafted with Dkk1/GFP expressing cells (B and D) or with GFP expressing cells (C and E). Grafts performed at sphere (B and C) or shield (D and E) stages. (F–K) Embryos grafted with Dkk1/GFP expressing cells (F, H, J, K) or with GFP expressing cells (G and I) at sphere (F, G) or shield (H, I, J, K) stages. Graft of cells expressing Dkk1 at shield stage is unable to rescue the headless phenotype (panel H and arrowhead in panel K) induced by *gsc/foxA3* knock-down, whereas it results in a loss of ventral mesoderm (arrowhead in panel J) showing that Dkk1 secreted from grafted cells was able to efficiently inhibit wnt8a activity even at long distance. (L) Frequency of phenotypic classes obtained at 24 hpf after the abovementioned transplant experiments. (B–E) Dorsal views at the 2–3 somite stage, (F–K) lateral views at 24 hpf.

set aside (Kuo et al., 1998; Nakata et al., 1997). It is not clear, however, when and how the neuroectoderm is induced and patterned.

Simultaneous inhibition of BMP and Wnt signaling was shown to induce head formation in frog embryos (Glinka et al., 1998; Glinka et al., 1997; Piccolo et al., 1999). Consistent with this, multiple Wnt, Nodal and BMP inhibitors were identified in discrete, overlapping domains of the organizer during gastrulation (De Robertis et al., 2000). These data correlate with the well known axis inducing property of the gastrula organizer (Spemann and Mangold, 1924) and support the initially accepted idea considering neural induction and patterning as gastrula events (Nieuwkoop et al., 1952).

However, Grinblat et al. (1998) showed that dorsal ectoderm explants are prespecified to express two regional forebrain markers, *opl* and *fkh5*, just before the onset of gastrulation (between 30 and 35% epiboly and shield stage). This indicates that the onset of forebrain patterning occurs earlier than gastrulation, but the molecular basis of the forebrain induction remained to be discovered.

In these regards, the expression of *gsc* and *foxA3* at the blastula stage raised the possibility of a wnt signaling inhibitory activity at these early developmental stages and we hypothesized that the headless phenotype obtained after *gsc* and *foxA3* inactivation may result from an exposure of presumptive anterior neural tissues to the caudalizing factor Wnt8a as soon as blastula stage. In agreement with our presumption, the transplant analysis presented here shows that the rescue of the head in the *gsc/foxA3* knock-down embryos is effective at the sphere stage whereas it is not effective anymore at the shield stage (Fig. 7). This supports the idea that *gsc* and *foxA3* act before the onset of gastrulation to prevent stimulation of presumptive anterior neural tissues by the caudalizing factor Wnt8a.

Previous studies suggested that formation of the nervous system involves at least three major steps. First, ectodermal cells must acquire neural identity, second rostrally positioned neural tissue must adopt anterior character and third regional patterning must take place within the neural plate (Wilson and Houart, 2004). However, there remains much controversy and discussion regarding the extent to which these events, particularly the first two, are independent or intrinsically linked to each other (De Robertis et al., 2000; Foley and Stern, 2001; Stern, 2002).

In many assays, whenever neural tissue is induced, it expresses transcripts that are later restricted to forebrain and midbrain territories raising the possibility of a link between induction of neural identity and acquisition of anterior character (Foley and Stern, 2001). In such a scenario, anterior neural identity appears to be the default state and formation of posterior structures requires the action of caudalizing factors.

In some experimental conditions, neural tissue apparently never passes through a phase of expression of anterior markers. For instance, frog ectodermal explants exposed contemporaneously both to neural inducing and caudalizing signals express posterior, but not anterior neural markers (Papalopulu and Kintner, 1996). As well, in double *gsc/foxA3* inactivation, upregulation of Wnt8 signaling at the dorsal margin results in a posteriorization concomitant with the induction of presumptive neural tissue and that can be fully rescued by the additional inactivation of *wnt8a*.

Altogether, here we demonstrate in vivo that the function of the transcription factors Gsc and FoxA3 is to promote formation of anterior neural tissue by protecting presumptive anterior neural cells from the caudalizing activity of Wnt8a. This protection is already set at early blastula stage, shortly after the initiation of zygotic genome expression, when wnt8a starts to be expressed. At this stage, cells of the presumptive anterior neural plate are located close from the dorsal margin. The activity of Gsc and FoxA3 at blastula stage prevents the expression of *wnt8a* and promotes expression of Wnt antagonists such as Frzb and Dkk1 in this territory. In consequence, they protect the presumptive anterior neural cells from a stimulation by Wnt8a, a necessary condition to avoid the irreversible caudalization of anterior neural tissue.

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